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DEVELOPMENT OF A BIOMONITORING ASSAY
FOR **CBI** OR ITS METABOLITES
IN HUMAN URINE

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GENERAL INFORMATION

Material Tested:

[CBI]

Synonyms:

[CBI]

Haskell Number:

[CBI]

Purity:

99.5%

Composition:

Pure compound

Contaminants:

Unknown

CAS Registry Number:

[CBI]

Sponsor:

DuPont Chemicals
E. I. du Pont de Nemours & Company, Inc.
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**DEVELOPMENT OF A BIOMONITORING ASSAY
FOR [CBI] OR ITS METABOLITES
IN HUMAN URINE**

SUMMARY

A GC/MSD assay was developed to measure the concentration of [CBI] a metabolite of [CBI] found in urine following the entrance of [CBI] into the system either by oral ingestion or skin contact. HPLC was evaluated as a technique for this measurement, but conventional detectors (UV, fluorescence) were found not to have the sensitivity required for this assay. The level of quantitation (LOQ) of the GC-MSD assay is below 100 parts per billion (100 µg/liter), the level needed to monitor significant exposure to [CBI]

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I. PURPOSE

The purpose of this project was to develop an analytical method that could be used to measure the level of exposure of a worker to **CBI** by monitoring either **CBI** or one of its metabolites in urine.

II. RECORDS

All raw data and the final report will be stored in the archives of Haskell Laboratory for Toxicology and Industrial Medicine, E. I. du Pont de Nemours & Company, Inc., Newark, DE, or in the Du Pont Records Management Center, Wilmington, Delaware.

III. MATERIALS AND METHODS

A. Test Substances

In addition to **CBI** several compounds expected to be metabolites of **CBI** were tested. See Figure 2. **CBI** was obtained from DuPont Chemicals; 4-Amino-m-cresol (97%), **CBI** (98%), **CBI** (97%) and **CBI** (99%) were obtained from Aldrich Chemical.

CBI and **CBI** were synthesized from **CBI** and **CBI** at the DuPont Glasgow Site by D. M. Simons of Medical Products. They were purified by recrystallization from water. **CBI** and from **CBI**. The identity of each material was confirmed with GC/MSD analysis.

B. Biological Methods

Metabolites of **CBI** were identified by dosing rats with **CBI** (500mg/kg oral dose) and chromatographing urine to collect fractions for subsequent identification of the major components by mass spectral analysis. All urine samples were incubated overnight with glucuronidase/sulfatase enzymes before HPLC analysis. The biological methods are described in HLR 166-93 (S. G. Hundley).

C. Mass Spectral Analysis

Fractions collected from the liquid chromatography discussed above were analyzed by particle beam electron ionization LC-MS for identification of the major component of the fractions eluting as a detectable peak in the chromatography of urine samples. The LC separation was done on a Hitachi instrument with an L6000/L6200 pumping system and a VG Biotech Quattro triple quadrupole instrument was used for the MS analysis.

D. HPLC Analysis

HPLC was the first technique considered for the development of a routine monitoring assay. Cheever et al. reported the detection of [CBI] in the alkaline hydrolysis product of a hemoglobin adduct with [CBI] using HPLC with fluorescence detection. [CBI] was identified in this work by GC-MS.¹ Peterson et al. used HPLC with electrochemical detection as a way of determining [CBI] in human urine.²

HPLC was evaluated as a technique for measuring the concentration of either [CBI] or one of the identified metabolites in urine. Both UV and fluorescence were investigated as a means of detection. Conditions used in this analysis are shown below:

Conditions for HPLC Analysis

Instrument:	
Column:	CBI
Mobil phase:	
Gradient:	CBI
Flow:	
Injection:	
Detection (UV):	CBI
Detection (fluorescence):	

Using these conditions with UV detection only, then with UV excitation and fluorescence emission detection, samples of urine from the rat studies were analyzed. Untreated controls were run and compared to treated samples at two levels of exposure. Comparison samples of the metabolites were run, using the reference standards available. Urine samples were diluted 1:1 with methanol and stored under refrigeration (about 4°C) until analyzed.

E. GC/MSD Analysis

GC/MSD has been used for the separation and measurement of several amines in various matrices, including urine. Bryant et al. used GC-MS as a quantitation method for 4-aminobiphenyl following mild basic hydrolysis and derivatization.³

GC/MSD was evaluated here as a possible means of measuring either the primary compound, [CBI] or one of its metabolites in urine. Conditions for this analysis were as follows:

Conditions for GC/MSD Analysis

Instrument:] CBI]
Column:	
Injection temperature:	
Injection volume:	
Heating rate:	
Detector mode:	

Urine samples were diluted 1:10 with methanol for analysis. As with HPLC, samples of urine from control animals were compared to urine from treated animals, and metabolites were analyzed for peak identification. The compounds of interest [CBI] all gave good chromatographic peaks and mass spectra clearly that of the expected compound when run under scanning mode. For greater sensitivity at low levels, SIM (selected ion monitoring) mode was used, searching only for the major mass peak of the analyte of interest.

Finally, a reproducibility study was done for the analysis of [CBI] analyzing the same sample twelve times.

IV. RESULTS AND DISCUSSION

A. Mass Spectral Analysis

The two principal metabolites identified by LC-MS in the excreted urine were [CBI]. In one fraction taken at about 18 minutes, [CBI] was also identified, along with [CBI]. Figure 1 shows the chromatogram of urine from treated rats. The last peak in the chromatogram, at 47.44 minutes, was not identified in the mass spectral analysis, however, this peak was not observed in urine from rats receiving a 1 mg/kg oral dose.

Mass Spectra from the analysis of these fractions are shown for the fractions collected at 18 and 34 minutes in Figures 3, 4 and 5, showing the presence of CBI in Figure 3, CBI in Figure 4 and CBI in Figure 5. CBI is evident in Figure 4 as the mass line at 123 m/z units. Compare these spectra to the spectra of standards for these compounds in Figures 13, 14 and 15.

B. HPLC Analysis

There was an obvious difference between controls and treated samples in the HPLC analysis with UV detection, but interpreting that difference was made difficult by the very complex nature of the chromatogram. See Figures 6 and 7. At higher concentrations (about 1600 µg/mL in undiluted urine), CBI appears as a small peak at 11.58 minutes in Figure 7 (see Figure 8 also for CBI), but at the concentrations expected at low exposure doses (about 12.8 µg/mL in urine before dilution), this peak virtually disappears (see Figure 9).

Of the two metabolites identified in the radiolabeled chromatogram, only one, CBI, shows up in the UV chromatogram, as a peak at 6.61 minutes (see Figures 7, 9 and 11), and this peak was too close to a peak present in the control sample to provide a basis for quantitation of this metabolite.

Although fluorescence gave cleaner chromatograms, the fundamental problem remained the same. The desired analytes at low levels gave peaks too small for adequate measurement and no fluorescence peaks could be detected when CBI was injected.

C. GC/MSD Analysis

The advantage of using GC/MSD for this analysis, at least during the development stage, is the ability to identify eluting components by examination of the mass spectra of peaks in the chromatogram. Figure 12 gives an example of this, where a reference sample of CBI was injected and the peak at 1.65 minutes was identified as the injected material by its mass spectra, which shows a molecular ion at mass 107, the molecular weight of CBI. Figure 2 shows the structure of CBI and some of the possible metabolites.

Following the biological testing and identification of two major metabolites, CBI and its acetylated analog, the work with GC was concentrated on those and CBI itself. Reference standards of the two metabolites were injected, giving the chromatograms shown in Figures 12 and 14. When these two metabolites were added to control urine and analyzed, the acetylated metabolite eluted with less interference from urine than CBI itself. Figure 16 shows the chromatogram resulting from the analysis of a sample after addition of 100 ppb of CBI to control urine.

D. Reproducibility

This sample (chromatogram shown in Figure 16) was analyzed 12 times to determine the reproducibility of the analysis. With a mean value for the peak area of 102,329, the standard deviation was 21,627 and the coefficient of variation (Cv) was 21.1%. This was higher than desired and further work was planned to improve this by using an internal standard, since it is believed that the major source of variability is in the size of the sample actually injected onto the column. The system uses a splitter, and even with an absolutely constant volume injected by the autoinjector, the amount split off will show some variation. The use of an internal standard should eliminate most of this source of variation. This approach is being evaluated by Paul Lieder at Haskell and will be reported in [CBI]

V. REFERENCES

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3. M. S. Bryant, P. L. Skipper, S. R. Tannenbaum and M. Maclure, "Hemoglobin Adducts of 4-Aminobiphenyl in Smokers and Nonsmokers", *Cancer Research*, **47**, (1987).

Figure 1

Chromatogram of Radiolabeled Metabolites

CBI

Figure 2

CFI and Potential Metabolites

CFI

* Identified as urinary metabolites in current study.

Figure 3

Mass Spectra of Fraction Taken at 19 Minutes

CSI

Figure 4

Mass Spectra of Fraction Taken at 19 Minutes Showing Second Component

CBI

Figure 5

Mass Spectra of Fraction Taken at 32.4 Minutes

CBI

Figure 6

HPLC Chromatogram of a Control Urine Sample

CBI

Conditions for HPLC Analysis

Instrument:

Column:

Mobil phase:

CBI

Gradient:

Flow:

Injection:

Detection (UV):

Detection (fluorescence):

Figure 7

HPLC Chromatogram of a Urine Sample at High Level Treatment

CSI

Conditions for HPLC Analysis

Instrument:

Column:

Mobil phase:

Gradient: CSI

Flow:

Injection:

Detection (UV):

Detection (fluorescence):

Figure 8

HPLC Chromatogram of [CBI]

CBI

Conditions for HPLC Analysis

Instrument:
Column:
Mobil phase:
Gradient:
Flow:
Injection:
Detection (UV):
Detection (fluorescence):



Figure 9

HPLC Chromatogram of a Urine Sample at Low Level Treatment

051

Conditions for HPLC Analysis

Instrument:

Column:

Mobil phase:

Gradient:

051

Flow:

Injection:

Detection (UV):

Detection (fluorescence):

Figure 10

HPLC Chromatogram of [CBI]

CBI

Conditions for HPLC Analysis

Instrument:

Column:

Mobil phase:

Gradient:

CBI

Flow:

Injection:

Detection (UV):

Detection (fluorescence):

Figure 11

HPLC Chromatogram of CRI

Conditions for HPLC Analysis

Instrument:
Column:
Mobil phase:

Gradient:

Flow:
Injection:
Detection (UV):
Detection (fluorescence):

Figure 12

GC/MSD Chromatogram of [CBI]

503

Conditions for GC/MSD Analysis

Instrument:
Column: CBI
Injection temperature:
Injection volume:
Heating rate:
Detector mode:

Figure 13

GC/MSD Chromatogram of CBI

Conditions for GC/MSD Analysis

Instrument:
Column:
Injection temperature:
Injection volume:
Heating rate:
Detector mode:

Figure 14

GC/MSD Chromatogram of CS 1

CS

Conditions for GC/MSD Analysis

Instrument:
Column:
Injection temperature:
Injection volume:
Heating rate:
Detector mode:

CS

0 0 2 5

Figure 15

GC/MSD Chromatogram of [] CBI

CBI

Conditions for GC/MSD Analysis

Instrument:
Column:
Injection temperature:
Injection volume:
Heating rate:
Detector mode:

Figure 16

GC/MSD Chromatogram of CS! in Control Urine

CS!

Conditions for GC/MSD Analysis

Instrument:
Column:
Injection temperature:
Injection volume:
Heating rate:
Detector mode:

CS!

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